

INDUCTION OF POLYPLOIDS AND ISOLATION OF PLOIDY VARIANTS THROUGH STOMATAL PARAMETERS IN BOUGAINVILLEA

(*BOUGAINVILLEA SPP*)

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ABSTRACT

Even though many varieties available in Bougainvillea, novelty in plant traits is appreciated to meet the demand of landscape industry. In this present investigation, anti-mitotic agents like colchicine and oryzalin were used to induce ploidy variations in two Bougainvillea cultivars of Lalbagh and Mahara. Of the putative polyploids of the cvs. Lalbagh and Mahara, plants with stomatal densities ranging between 13.00 and 22.00, 10.00 and 21.00 stomata mm⁻² and stomata size between 78.93 and 159.32, 78.93 and 169.56 μm² respectively were categorized as diploids whereas those with stomatal densities between 5 and 9 stomata mm⁻² and stomatal size between 176.20 and 672.98, 182.42 and 398.09 μm² respectively were categorized as tetraploids. The proportion of various ploidy levels based on the stomatal traits revealed that 0.4 % colchicine recorded the highest rates of tetraploids (26.67 %) and 100 μM oryzalin recorded the highest rates of mixoploids (27.78 %) in cv. Lalbagh. In case of Mahara, the highest rate of putative tetraploids (16.67 %) was observed in 0.3 % colchicine and 100 μM oryzalin, whereas 0.3 % colchicine recorded 11.11 per cent of mixoploids.

KEYWORDS: Anti-Mitotic Agents, Bougainvillea, Colchicine, Oryzalin, Polyploidy and Stomata

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INTRODUCTION

Bougainvillea (*Bougainvillea spp.*) is an ornamental plant belonging to the family Nyctaginaceae and it is native to winter dry tropical region of South America. The genus includes the showiest ornamentals of tropics and subtropics and now they have become an important part of the tropical horticulture. Its profuse and round-the-year flowering nature, potential to withstand diverse climatic conditions and adverse situations including drought, salinity, alkalinity and urban pollution due to its hardy nature and its attractive bracts produced in a wide array of colours. The additional merits of Bougainvillea are its low water and nutritional requirements and freedom from pests and diseases as well as pollutant resistant (Shiragave *et al.*, 2015). Bougainvillea possesses a great demand in the landscaping industry, development of home gardens, factory gardens, municipal gardens, airport surroundings, five star hotels, holiday resorts, newly built townships, historical monuments, temple surroundings and road side plantations (Banerji *et al.*, 2008). Because of the great demand of Bougainvillea in landscaping purpose, creation of novel variation in the foliage, bract and stem are of prime importance.

Polyploidy is considered to be a major pathway for plant evolution and can contribute to reproductive isolation and abrupt speciation (Wendel, 2000; Soltis *et al.*, 2003). The effects of polyploidy on plant traits are also

important to horticulturists and plant breeders for crop improvement. Since ploidy breeding is the easiest and effective method in creating variation in vegetatively propagated crops, the present study was undertaken with an aim of inducing desirable and novel variations in *Bougainvillea* cultivars viz., Lalbagh and Mahara through the use of anti-mitotic agents (colchicine and oryzalin) and identification of desirable putative polyploids. In this study, to know the response of anti-mitotic agents (Colchicine and Oryzalin) on both multibracted and single bracted types, Lalbagh and Mahara were employed. More over Mahara being multibracted and devoid of true flower, ploidy breeding would be an effective method in creating variations. For screening of putative polyploids morphological as well as stomatal traits can be very well utilized. Root tip cell chromosome counting is time consuming and not suited for routine screening of ploidy level. For this purpose, the stomatal cell length can be a rapid and reliable indirect ploidy indicator (Mishra, 1997). Stomatal frequency, guard cell length and stomatal plastid number have often been used as morphological marker for identifying ploidy levels in many plant species (Beck *et al.* 2003, Yuan *et al.* 2009, Ye *et al.* 2010). In general, stomata and epidermal cell frequency per unit leaf area diminished while stomata guard cell length improved with an increase in ploidy (Yuan *et al.* 2009, Ye *et al.* 2010). Therefore, this study focuses on the isolation of ploidy variants via stomatal parameters using microscopic examination.

MATERIAL AND METHODS

The ploidy work in *bougainvillea* using anti-mitotic agents was carried out at the Department of Floriculture and Landscaping, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore (Latitude of 11°00'N, Longitude of 77°00'E and an elevation of 412 m above MSL), Tamil Nadu during August 2014 to Aug 2016. Hardwood cuttings of diploid ($2n=2x=34$) *Bougainvillea* cultivars Lalbagh (single bracted) and Mahara (multi-bracted) were utilized as biological material and obtained from the germplasm collection maintained at Department of Floriculture and Landscaping. Lalbagh and Mahara belongs to the Red group (51-A) and Red Purple group (70 A) of the Royal Horticultural Colour chart. Colchicine and Oryzalin were employed as anti-mitotic agents for chromosome doubling in both cultivars.

Mode of Treatment

Hardwood cuttings (13-15 cm long and pencil thickness) of both the cultivars were planted in polybags and kept in mist chamber for rooting. Immediately after the emergence of dormant buds in the nodal regions, cotton plugs saturated with different concentrations of colchicine (0.1, 0.2, 0.3 and 0.4%) and oryzalin (50, 100, 150 μ M) solutions were placed intact on the sprouts with the help of cellophane tape for 72 hours. The cotton plugs were resoaked with the chemicals when required. These cuttings were covered using polycaps to avoid contact with water in the mist chamber. In each treatment of Colchicine and Oryzalin, twenty plants per treatment were selected for screening using stomatal traits. In each selected plant, leaf pair in the fifth nodal region of all anti-mitotic agent treated branches were collected and subjected to stomatal analyses for the verification of ploidy status under microscopic examination.

Estimation of Stomatal Characters

The number of stomata mm^{-2} in the leaves has been reported to be proportionate to the ploidy level upto pentaploidy (Sathiamoorthy, 1973). The standard stomatal density values for different ploidy levels as assessed by Sathiamoorthy (1973) and stomata size as per the reports of Vandenhout *et al.* (1995) have been taken to assess the ploidy status of the regenerated plantlets. For assessment of stomatal density and size, impression of the abaxial surface of the leaf

was taken using a solution prepared by dissolving thermocole in xylene and the impression was observed under microscope. Stomatal density was measured in the abaxial side of leaves and expressed as number of stomata mm^{-2} . Stomatal size was measured as area of stomata (Length x Breadth) and expressed as μm^2 .

RESULTS AND DISCUSSIONS

The stomatal traits of the colchicine and oryzalin treated branches of Lalbagh and Mahara are presented Table 1. The leaves from individual branches of the anti-mitotic agents treated cuttings were analysed under microscope to determine the density and size of the stomata (Figure 1) to identify its ploidy level. Plants with stomatal densities ranging between 13.00 and 22.00 mm^{-2} and stomata size between 78.93 and 159.32 μm^2 were categorized as diploids in Lalbagh whereas stomatal densities ranging between 10.00 and 21.00 stomata mm^{-2} and stomata size between 78.93 and 169.56 μm^2 were categorized as diploids in Mahara. In Lalbagh, those with stomatal densities between 5 and 9 stomata mm^{-2} and stomatal size between 176.20 and 672.98 μm^2 were categorized as tetraploids and stomatal densities between 5 and 9 stomata mm^{-2} and stomatal size between 182.42 and 398.09 μm^2 were categorized as tetraploids in Mahara. Plants which had mixtures of two or more of these stomatal densities and dimensions were categorized as mixoploids.

Earlier studies of induced and spontaneous polyploid plants have frequently observed that stomatal size, stomatal number and the number of chloroplasts within the guard cells varied significantly in the event of chromosome doubling, compared with the diploid state (Nigel *et al.*, 2007). Abak *et al* (1998) reported in many of plant species, there are correlation between ploidy level and cytogenetic characteristics such as chloroplast number in guard cells, size of stomata cells, stomata density and pollen grain diameter. Stomatal characteristics (stomatal size and density) have previously been used as parameters for distinguishing ploidy in the Orchidaceae, for the orchids *Cymbidium* (Wimber and Van Cott, 1966), *Paphiopedilum* (Watrous and Wimber, 1988), *Phalaenopsis* (Chen *et al.*, 2009), and other orchid species (Miguel and Leonhardt, 2011). Putative tetraploids of *Zantedeschia* derived through colchicine treatment have been screened for ploidy based on stomatal length, demonstrating that stomatal length is a good indicator of ploidy level in *Zantedeschia* (Cohen and Yao, 1996).

Stomata size is another parameter that can be used as a tool to prescreen for polyploids. This is in agreement with results from *Xanthosoma* (Tambong *et al.*, 1998), *Alstromeria* (Lu and Bridgen, 1997) and *Scutellaria baicalensis* (Gao *et al.* (2002) that stomata in polyploids were found to be significantly larger than diploids. The utility of stomata size in distinguishing plants with different ploidy has been demonstrated in other plant types (Campos *et al.*, 2009). The average stomatal size of the tetraploids was 2.09 and 1.95 times as that of diploids in *Bougainvillea* cultivars Lalbagh and Mahara respectively. The stomatal density in diploid plants of Lalbagh and Mahara was 1.85 and 1.96 times greater than that observed in tetraploid plants, respectively.

Similar findings were also reported by Sakhanokho *et al.* (2009) in ornamental ginger with respect to stomatal frequency in diploid plants which had 1.8 times greater than that observed in tetraploid plants. The results pertaining to stomatal size are in agreement with results from *Xanthosoma* (Tambong *et al.*, 1998), *Alocasia* (Thao *et al.*, 2003), and *Alstromeria* (Lu and Bridgen, 1997) where stomata in polyploids were found to be significantly larger than diploids. Compared to the diploid plants, which had an average of 39 stomata per mm^2 , tetraploid plants had a much lower stomatal density of only about 22 stomata per mm^2 .

Polyploid plants often have negative relationships between stomata size and stomata density (Khazaei *et al.*, 2010;

Miller *et al.*, 2012), and the regulation mechanisms between stomata size and stomata density are not known yet. Increased numbers of chromosomes in polyploids often changes a lot of gene expressions, resulting in phenotypic alteration including stomata density in plant (Comai, 2005). The regulation mechanisms of stomata density on the molecular level are still unrevealed.

Ploidy Levels of Regenerants (%)

The percentage of different ploidy levels of treated cuttings was assessed based on the stomatal analyses through microscopic examination and presented in Table 2. The highest rates of tetraploids were obtained in 0.4 % and 0.3 % colchicine with respect to Lalbagh, whereas, in Mahara, 100 μ M oryzalin and 0.3 % colchicine observed with higher percentage of tetraploids (16.67 %). Higher rates of mixoploids were registered in 100 μ M oryzalin concentration of Lalbagh and 0.1 % colchicine in Mahara. The results obtained in Mahara are lined with the findings on induction of tetraploidy using colchicines and oryzalin in ornamental ginger

Table 1: Effect of Anti-Mitotic Agents (Colchicine and Oryzalin) on Stomatal Traits of Bougainvillea cvs. Lalbagh and Mahara

Treatment	Ploidy level	Lalbagh				Mahara			
		Stomatal density (number mm ⁻²)		Stomatal size (μ m ²)		Stomatal density (number mm ⁻²)		Stomatal size (μ m ²)	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean
Absolute control	2n	13 - 21	16.00	112.35 - 154.14	128.95	13 - 21	14.56	115.25 - 146.11	128.44
	4n	-	-	-	-	-	-	-	-
0.1 % Colchicine	2n	12 - 17	13.06	93.35 - 148.14	134.50	11 - 15	12.71	97.96 - 162.25	127.14
	4n	7 - 9	8.00	278.5 - 672.98	378.75	5 - 8	6.00	308.78 - 398.09	353.44
0.2 % Colchicine	2n	10 - 16	12.88	101.05 - 144.07	125.91	10 - 15	12.60	104.57 - 169.56	137.48
	4n	7 - 8	7.67	197.48 - 298.67	234.99	5 - 8	6.33	261.89 - 388.48	311.81
0.3 % Colchicine	2n	12 - 17	13.25	110.76 - 153.39	134.37	10 - 16	12.45	98.04 - 156.09	131.02
	4n	5 - 8	6.25	198.68 - 320.62	258.69	5 - 8	7.17	227.27 - 307.67	258.09
0.4 % Colchicine	2n	12 - 15	13.17	111.24 - 157.60	130.56	8 - 17	12.10	108.18 - 158.18	134.42
	4n	6 - 9	7.67	176.20 - 255.21	201.46	5 - 6	5.50	224.07 - 250.00	237.04
50 μ M Oryzalin	2n	10 - 15	12.58	104.34 - 148.50	126.45	10 - 15	13.14	114.91 - 159.67	145.62
	4n	6 - 8	7.40	183.40 - 292.19	208.00	7 - 9	8.00	195.88 - 236.95	216.42
100 μ M Oryzalin	2n	11 - 22	15.79	78.93 - 159.32	133.34	10 - 15	12.43	78.93 - 169.32	126.45
	4n	8 - 9	8.20	193.09 - 221.06	378.75	5 - 8	6.67	246.57 - 274.12	258.09
150 μ M Oryzalin	2n	12 - 21	16.58	105.89 - 154.45	123.03	10 - 13	12.00	121.25 - 161.95	141.40
	4n	8 - 9	8.30	189.28 - 391.68	230.56	5 - 7	6.00	182.42 - 189.37	185.90

Table 2: Proportion of Various Ploidy Levels of Regenerants Derived from Anti-Mitotic Agent Treated Cuttings of Bougainvillea cvs. Lalbagh and Mahara by Stomatal Analyses

Treatment	Lalbagh			Mahara		
	% (2n)	% (4n)	(Mixoploids)	% (2n)	% (4n)	(Mixoploids)
Absolute Control	100.00	-	-	100.00	-	-
0.1 % Colchicine	83.34	5.55	11.11	79.47	10.53	10.00
0.2 % Colchicine	75.00	15.00	10.00	85.00	12.50	6.25
0.3 % Colchicine	80.00	18.75	1.25	72.22	16.67	11.11
0.4 % Colchicine	60.00	26.67	13.33	76.93	15.38	7.69
50 μ M Oryzalin	64.71	11.76	23.53	90.00	5.00	5.00
100 μ M Oryzalin	61.11	11.11	27.78	75.00	16.67	8.33
150 μ M Oryzalin	69.23	7.69	23.08	93.33	6.67	-

(Sakhanokho *et al.*, 2009) wherein oryzalin was effective in inducing polyploidy at all concentrations and

percentages of induced tetraploidy increased for 30 mM and 60 mM oryzalin but decreased for 120 mM oryzalin. In Alocasia, oryzalin had an effect over a wide range of concentrations and resulted in more polyploid materials (Thao *et al.*, 2003). High concentrations of colchicine have been associated with plant cell death because of the highly toxic effect, which blocks spindle fiber development and modifies the differentiation process (Pintos *et al.*, 2007). This toxicity is in part attributable to the generally higher colchicines concentrations needed for polyploidy induction because colchicine binds poorly to plant tubulins (Morejohn *et al.*, 1987).

A comparison of the polyploidization efficiencies of the two anti-mitotic agents in the present study indicated that to obtain tetraploid rates on par with oryzalin around 75 times the concentration of colchicines would be required. Overall, the results showed that oryzalin was more efficient in inducing polyploidy, in particular tetraploidy, than colchicine in Mahara. The results obtained are in accordance with the findings of Denaeghel *et al.* (2015) in Escallonia, in which 33.3% *E. rosea* tetraploids were obtained using oryzalin (150 μ M for 3 days), compared to colchicine which only yielded 16.7% *E. rubra* (1000 μ M for 3 days) and 3.3% *E. rosea* (2000 μ M for 3 days) tetraploids.

The variability of the ploidy level within the same treatment condition could be due to the stage of the cells that were responsible for initiating new shoots (Thao *et al.*, 2003). Although a number of cells in meristematic tissue may become polyploidized, many others may be unaffected and remain diploid. Thus, the areas where both normal and polyploidized cells are intermixed may be termed 'mixoploidy' or so called 'chimera' (Dermen and Henry, 1944; Pryor and Frazier, 1968; Wan *et al.*, 1989). The large number of chimeric plants is mainly due to the type of tissue used. In multicellular buds or meristems only a portion of the cells or cell layers may be affected, while the other portion remains diploid (Pryor and Frasier, 1968). Instead, if single cells are treated, the result is a plant with only one ploidy level.

CONCLUSIONS

The study concluded that, both the anti-mitotic agents namely Colchicine and Oryzalin generates novel putative polyploids in treated buds of Bougainvillea at higher percentage. Stomatal parameter analysis accomplished that, tetraploid and mixoploids numbers were maximum at higher concentration of Colchicine (0.3 % and 0.4%) and Oryzalin (100 μ M) in both varieties of Lalbagh and Mahara. In case of Lalbagh, oryzalin produces higher number of mixoploids than colchicines whereas colchicine is effective for the generation of polyploids (tetraploids and mixoploids) in Mahara. This shown varietal difference of the genotype used for anti-mitotic agent treatment also creates variation in the ploidy level of emerging new shoots in Lalbagh and Mahara. This fundamental study on ploidy generation would be more effective for the selection of optimum concentration anti-mitotic agent in the development of new novel variants in Bougainvillea.

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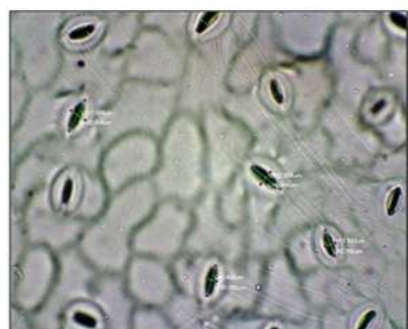
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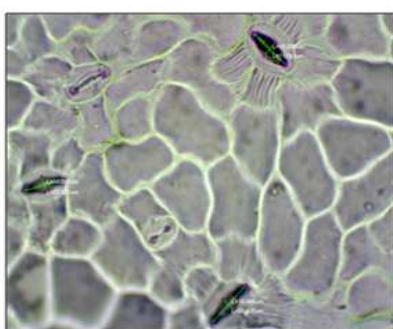
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APPENDICES

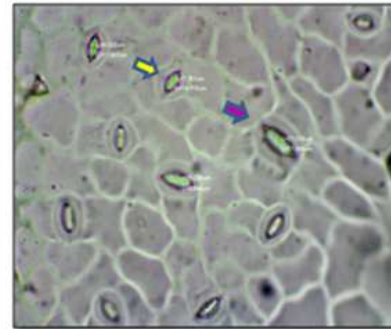
A. Lalbagh



Control – Diploid (2X)

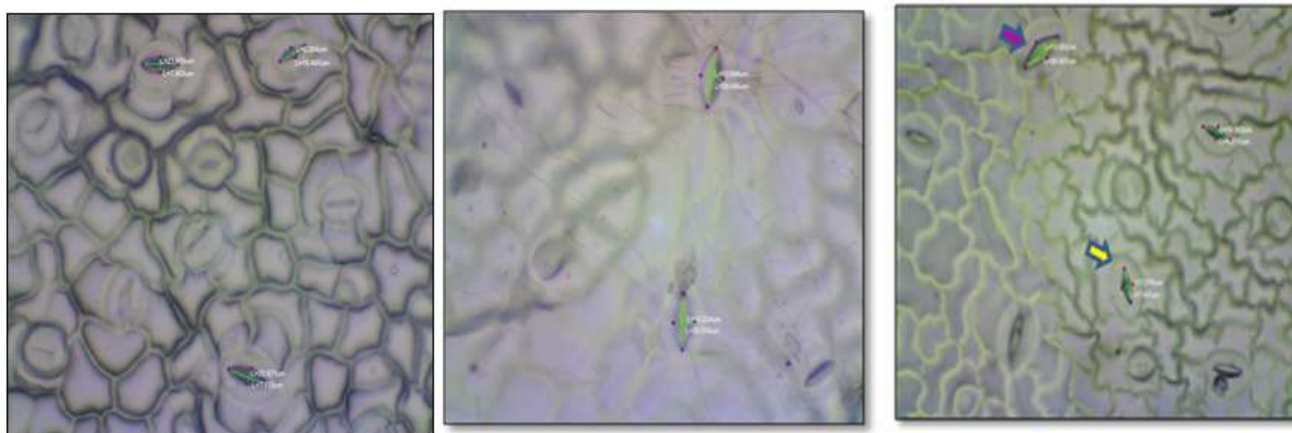


Tetraploids (4X)



Mixoploids (2x+4x) (Yellow – 2x and Purple – 4x)

B. Mahara



Control – Diploid (2X)

Tetraploids (4X)

Mixoploids (2x+4x) (Yellow – 2x and Purple – 4x)

Figure 1: Variation in Stomatal traits (Density and Size) in Putative Polyploids of cvs. (A) Lalbagh and (B) Mahara